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Cyclophilin D over-expression increases mitochondrial complex III activity and accelerates supercomplex formation

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ABSTRACT

Cyclophilin D (CyPD), a mitochondrial matrix protein, has been widely studied for its role in mitochondrial-mediated cell death. Unexpectedly, we previously discovered that overexpression of CyPD in a stable cell line, increased mitochondrial membrane potentials and enhanced cell survival under conditions of oxidative stress. Here, we investigated the underlying mechanisms responsible for these findings. Spectrophotometric measurements in isolated mitochondria revealed that overexpression of CyPD in HEK293 cells increased respiratory chain activity, but only for Complex III (CIII). Acute treatment of mitochondria with the immumosupressant cyclosporine A did not affect CIII activity. Expression levels of the CIII subunits cytochrome *b* and Rieske-FeS were elevated in HEK293 cells overexpressing CyPD. However, CIII activity was still significantly higher compared to control mitochondria, even when normalized by protein expression. Blue native gel electrophoresis and Western blot assays revealed a molecular interaction of CyPD with CIII and increased levels of supercomplexes in mitochondrial protein extracts. Radiolabeled protein synthesis in mitochondria showed that CIII assembly and formation of supercomplexes containing CIII were significantly faster when CyPD was overexpressed. Taken together, these data indicate that CyPD regulates mitochondrial metabolism, and likely cell survival, by promoting more efficient electrons flow through the respiratory chain via increased supercomplex formation.
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1. Introduction

Mitochondria are major regulators of both cell death and cell survival [1–3]. The latter function is primarily attributed to the production of ATP, the final product of a coordinated action of five protein complexes in the mitochondrial respiratory chain (RC). As is well known, electrons are liberated by the oxidation of NADH and FADH2 within four consecutive complexes and two mobile carriers (coenzyme Q and cytochrome *c*), ultimately being transferred to oxygen. This generates the classic proton gradient across the inner mitochondrial membrane, which can then be used to drive ATP synthesis via the F_0F_1-ATP synthase (complex V). More recent work has shown that respiratory complexes can be organized, as functional entities, into supramolecular structures commonly referred to as "supercomplexes" [4]. The precise composition of complexes and supercomplexes appear to vary according to tissue type and temporal bioenergetic demands [5]. However, the overall impact of supercomplexes appears to be increased efficiency of electron transfer [6,7].

Complex III (coenzyme Q-cytochrome *c* reductase) anchors the central position in the RC, mediating electron transfer from ubiquinol to cytochrome *c*. It consists of 11 subunits, three of which constitute a functional core: cytochrome *b* (Cytb), cytochrome *c*1 (Cyt1) and the Rieske-FeS protein Rip1. Biogenesis of complex III (CIII) occurs in a modular step-wise assembly pathway. The subunit Cytb seeds the early core of the complex, which is subsequently

Abbreviations: CyPD, Cyclophilin D; CIII, Complex III; RC, mitochondrial respiratory chain; Cytb, cytochrome *b*; Cyt1, cytochrome *c*1; ROS, reactive oxygen species; mPTP, mitochondrial permeability transition pore; PPIases, peptidyl-prolyl*cis-trans*isomerases.

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stabilized by the incorporation of the Rip1 subunit [8].

A key event in necrotic cell death is the increased permeability of the mitochondrial inner membrane (MIM) [9], due to the functionally defined opening of the mitochondrial permeability transition pore (mPTP), a high conductance channel whose precise composition is unknown, but which includes, critically, cyclophilin-D (CyP-D) [10,11]. This is a mitochondrial isoform belonging to the highly conserved peptidyl-prolyl*cis-trans*isomerases (PPIases) family of proteins. Cyclophilins catalyze isomerizations around Xaa-Pro peptides, which are, in general, rate-limiting steps of protein folding [12]. Treatment of cells with the pseudo-CyP-D substrate cyclosporin A is widely known to inhibit cell death stimuli, which gives rise to the model that CyP-D sensitizes opening of the mPTP [13]. This model of cell death was constricted to necrosis based on the insensitivity of CyP-D null mice to high loads of mitochondrial calcium (Ca^{2+}), which did not impact their ability to undergo apoptosis [14]. Unexpectedly, our group discovered that overexpression of CyP-D in HEK cells decreased their resistance to cell death stimuli [15]. We concluded that CyP-D, like mitochondria, played a role in both cell death and cell survival [15].

More recent work has implicated many CyPs as key players in larger protein complexes, although their functional significance is not yet completely understood [16,17]. The goal of the work presented here was to test the hypothesis that CyP-D is an important regulator of mitochondrial metabolism. We present evidence showing that overexpression of CyP-D in HEK293 cells significantly increases the efficiency of CIII activity itself. This increase in activity appears to be independent of PPIase enzymatic activity, at least acutely. Moreover, we find that the assembly of both CIII and supercomplexes are accelerated by CyPD overexpression. We note that others have reported CIII is present in all supercomplexes and is considered the main stabilizing complex [18–24]. Taken together, these findings suggest that CyP-D enhances cell survival by increasing the efficiency of mitochondrial respiration via CIII and its central role in supercomplex activity.

2. Material and methods

2.1. Cell culture and growth conditions

Cells were cultured according to Lin and Lechleiter [15]. Briefly, HEK293 cell were maintained at 37 °C in Dulbecco's modified Eagle's medium/F-12 (Life Technology, Rockville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 200 μg penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air. The generation of a stable HEK293 cell line expressing CyPD was performed according to Lin and Lechleiter [15].

2.2. Mitochondrial isolation from HEK293 cells

Mitochondria from HEK293 cells were isolated according to the method described by Attardi et al. [25]. Briefly, HEK293 cells were scraped down in NKM solution (in mM: 130 NaCl, 5 KCl, 1 $MgCl_2$) and spun down at 250 g for 5 min. Pellets were incubated in TKM buffer (in mM: 10 Tris-HCl pH 6.7, 10 KCl, 0.15 $MgCl_2$). All the procedures were performed at 4 °C. The suspensions were manually homogenized (~50 strokes), transferred into a tube with 0.5 ml of Sucr-TKM buffer (in mM:10 Tris-HCl pH 6.7, 10 KCl, 0.15 $MgCl_2$, 2000 Sucrose) and centrifuged at 1000 *g* for 5 min. The subsequent supernatant was collected and centrifuged at 10,000 *g* for 10 min to obtain mitochondria pellets. The pellets were then resuspended with Suc-Tris-EDTA buffer (in mM: 10 Tris-HCl pH 6.7, 1 EDTA, 250 Sucrose) and centrifuged at 10,000 *g* for 10 min. TM-Suc buffer (in mM: 10 Tris-HCl pH 6.7, 0.15 $MgCl_2$, 250 Sucrose) was added to the mitochondrial pellets and spun at 10,000 *g* for 10 min to obtain the final mitochondrial pellet.

2.3. DDM solubilization

1 mg of mitochondria protein from HEK293 cells were solubilized in 1% DDM (Sigma Aldrich, St. Louis, MO) for 45 min at 4 °C on a rotator. Afterwards, the solubilization mix was spun at 72,000 *g* for 30 min. The supernatants were collected and benzonase treated (Sigma Aldrich, St. Louis, MO) for 30 min. The solubilized mitochondria supernatant was collected and aliquoted for Blue native gel electrophoresis or for SDS-PAGE analysis (12%) gels.

2.4. Activities of the respiratory chain complexes (ETCs) I–V

The activities of the electron transport complexes were measured by spectrophotometric assay. 10 μg of mitochondrial proteins in respiration buffer (in mM: 250 sucrose, 10 KH_2PO_4, 10 Tris-ClH, pH 7.4, 1 EGTA) were used to measure activity for complex I–IV. The activity for complex I was determined by monitoring the oxidation of NADH at 340 nm at 30 °C, using ubiquinone-2 as an electron acceptor in the presence of 2, 6 -dichlorophenolindophenol (DPIP) [26]. CII activity was measured by the succinate-dependent reduction of DPIP. The reaction was monitored at 600 nm at 30 °C using ubiquinone-2 as an electron acceptor [27]. CIII activity was measured by reduction of Cytochrome *C* Fe^{3+} using Decylubiquinol-2 as an electron donor at 550 nm. CIV activity was determined by monitoring the oxidation of cytochrome *c* Fe^{2+} at 550 nm. The data dimensions are μmol/min/mg protein.

2.5. SDS–page and western blotting

Electrophoresis was performed in SDS-polyacrylamide gels. Proteins were then either stained with Coomassie brilliant blue G-250 or transferred into PVDF membrane and probed with the following antibodies: anti-Core II, anti-Rieske (MitoProfile, Eugene, OR), polyclonal rabbit anti-CyPD antibody (custom made by Pocono Rabbit Farm & Laboratory, Inc. Canadensis, PA).

2.6. Blue native gel electrophoresis

Solubilized mitochondrial extracts (100 μg) were loaded onto a Native Page BisTris 4–12% gradient gel with Coomassie G250 (Life Technology, Carlsbad, CA) and run at 150 V using dark blue cathode buffer as directed by the manufacturer's protocol with Native Mark standards (Bovine heart) in the cold room. Staining of the Native Page gel was performed using Colloidal Blue Staining kit (Life Technology, Carlsbad, CA) following the manufacturer's protocol.

2.7. Blue native gel transfer

Blue Native transfer was achieved by using the iBlot Dry Blotting transfer system (Life Technology, Carlsbad, Ca) on setting P3 for 7 min transfer time. Gels were immersed in 2X transfer buffer prior to transfer for 10 min on shaker at room temperature. During the last five minutes of shaking, a final 0.1% SDS was added to the 2X transfer buffer. The gel was then loaded onto a PVDF membrane, transferred and fixed with 8% acetic acid and rinsed with dH20 prior to blocking and immunodetection.

2.8. 2-D electrophoresis of native page gel and transfer

After blue native gel electrophoresis and overnight distaining, the gel strips were treated following the manufacturer's protocol (Life Technology, Carlsbad, CA). Briefly, the gel strips were

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